Diversity and Molecular Systematics of Cone Snails (Family: Conidae) in the Karnataka Coast and the Lakshadweep Archipelago and Characterization of Conopeptides from *Conus biliosus* Venom

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CHAPTER 01: Introduction and review of literature.

1.1. Introduction

One of the most species-rich groupings of marine gastropod molluscs is the family Conidae J. Fleming, 1822; commonly referred to as cone snails which is a varied and highly specialized group of predatory marine gastropods. The genus *Conus*, holds large group of species from this family and has the most significant species as member of this family. Being able to capture prey with exceptional effectiveness is made possible by their highly sophisticated venom delivery mechanism and unusual, conical-shaped shells. Cone snails are distinguished by their tall, conical shells, which often have appealing variety patterns and colour. *Conus* spp., the shell shape may range in size from a few centimetres to over 26 cm. The spire in various species may range in height from low to high, and the aperture is small. The general body layout of cone snails is relatively constant between species, despite the diversity of shell morphologies. In general, Cone snails shells exhibit large variety of colors and patterns and different form in the same species. Shell size of these snails varies in size from species to species.

Their habitat ranges from intertidal zones to deep seas, and they are found throughout the world (Röckel et al., 1995). Cone shells are recorded in all tropical and subtropical oceans, although they are predominant in the Indo-West Pacific range and observed in a variety of maritime habitats, such as seagrass beds, rocky substrates, sandy bottoms, and coral reefs. Although the majority of species are marine, some have evolved to live in deeper ocean conditions; species have been found at depths of more than 1,000 meters from shoreline and intertidal zones.

Cone snail research has made substantial contributions to several scientific domains, most notably neuroscience, pharmacology, and toxicology. Conotoxins are useful instruments for studying ion channels and neurotransmitter systems, which has significance for medication development because to their exceptional potency and specificity. Cone snails are a topic of ongoing scientific interest because of the venom's continued ability to provide light on neuropharmacology and the possibility of new medicinal uses. Cone snails are a group of considerable interest to scientists and naturalists alike because of their remarkable predatory adaptations, variety of habitats, and enormous biological potential.

Despite the vast distribution of many cone snail species, habitat degradation, especially in coral reef environments, has made some species vulnerable. Threat to their populations also come from human activities like pollution and coastal development, as well as from ocean acidification and climate change. Cone snail conservation activities often form a component of larger programs to safeguard marine environments, particularly coral reefs and tropical settings where the snails are most prevalent. Cone snails have an indirect advantage from conservation initiatives that save coral reef ecosystems and marine biodiversity. Because these predatory gastropods are essential to the health of the reef ecosystem, efforts to preserve reef habitats also support the survival of these species. Cone snails also have cultural value in certain areas in addition to their biological and ecological relevance. Their exquisitely designed shells have been traded for generations and are highly prized by collectors. Cone snails are also utilized commercially as an ornamental art material. However, worries regarding overexploitation in certain places have arisen as conservational matter of concern.

1.2. Review of Literature

Distribution and Taxonomy

According to World Register of Marine Species (WORMS, Accessed on October 2024; Puillandre and Tenorio 2017) over 900 recognized species of cone snails (genus *Conus*) in different ocean waters. As taxonomists continue to contribute and resolve the cryptic species categories based on fresh morphological and genetic evidence, this number might fluctuate. Recently, Tucker & Tenorio, 2009; published an alternative shell and radula-based categorization that acknowledges 80 taxa and four families of cones. According to Puillandre et al. (2014), all cone snails may be classified into the family Conidae, which has four genera: *Conus*, *Conasprella*, *Profundiconus*, and *Californiconus*. This classification is based on molecular phylogenetic analysis. Approximately 85% of all recognized species are included in the genus *Conus* alone. But overall cone snails are divided into 04 genera and 71 subgenera.
The conventional approach of recording the variety of molluscan comprises taxonomic hypothesis testing using all available data, including anatomy, biogeographic information, and more recently, DNA molecular investigations. Finding and formalizing reliable differentiating variables requires a thorough examination of the shell features for every taxonomic determination made in malacology. The taxonomic research is using molecular approaches more often these days, and they are discovering a large number of cryptic species in all categories, including molluscs through genetic data and anatomical features (Williams & Reid, 2004; Collin, 2003; Duda et al., 2009).

To achieve this the intraspecific variability of the shell has to be assessed and quantified. There have been many taxonomic changes made to the Conidae family. *Conus* was the only genus into which cone snails were formerly classified, but molecular phylogenetic analyses have led to the suggestion that the group be divided into many genera to better represent their variety in evolution, continuing to be the most prevalent and speciose group. Fossil data demonstrate that cone snails have existed since the Eocene era and have preserved their unique form over millions of years. They are a crucial group to comprehend the development of marine biodiversity and predation tactics because of their remarkable variety and lengthy evolutionary history (Kumar et al., 2022).

Morphometry

Gastropod as the largest group, molluscs make up the second most populous phylum of animals after the arthropods and before chordates. There are currently between 70,000 and 76,000 species known to science, and over 200,000 estimated live species (Halanych 2004). Research in systematic biology have made use of geometric morphometric approaches, which enable the direct measurement and study of variances in biological form. (Cruz et al., 2012). Morphological complexity differences impact taxonomy and the rebuilding of evolutionary patterns, leading to significant implications, particularly in paleontology. Morphological identification of biological species has garnered considerable interest from paleontologists (Jackson and Cheetham 1990; Jablonski 1999). Taxonomic classification and the comprehension of evolution have historically relied on the morphological descriptions of organisms. Regardless of recent developments in molecular techniques, comparing anatomical and structural features of organisms continues to be a fundamental aspect of (paleo)biology. Variations in morphological complexity among taxa present challenges when species assignments rely exclusively on morphology. Specifically, a higher taxon with fewer morphological features is expected to yield a reduced number of lower-level taxa assignments (Schopf et al., 1975; Schopf 1982).

Throughout development stages, the skeletons of many species continue to expand. Even while a shell might be thought of as a single static shape, the morphology as a whole is the culmination of the continuous process of accretion. Because of this, a clearly ontogenetic description of form sheds light on the ultimate form that is produced. An analysis of landmark transformations provides direct access to the main morphological variation components in adult individuals as well as in the ontogeny of an individual. Morphological variation may also be characterized by parameters of predefined, abstract geometric models; however, there is no assurance that these parameters will match with the main characteristics of form variation (Johnston et al., 1991).

Molecular taxonomy

37 genes encoding 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs), and 13 protein subunits of the oxidative phosphorylation enzymes are found in the majority of metazoan mitochondrial genomes (Boore, 1999). The mitochondrial genomes of insects and vertebrates are well understood, while those of other species have received less research. Comparing the mitochondrial genomes of molluscs to those of other species, however, has

shown significant differences (Boore, 1999; Kurabayashi and Ueshima, 2000; Boore et al., 2004; Grande et al., 2006). Thirteen complete mitochondrial genomes of neogastropods have been discovered so far (Bandyopadhyay et al., 2006; Bandyopadhyay et al., 2008; Cunha et al., 2009; McComish et al., 2010; Simison et al., 2006). It's interesting to note that there are just two instances of tRNA gene translocations in these genomes' extremely conserved gene organization (Cunha et al., 2009). Animal mitochondrial genomes are often arranged in a highly ordered fashion. They have brief intergenic regions, and there may even be gene overlap with nearby genes. A lengthy non-coding mtDNA region, on the other hand, may be several kilobases (kb) long. This area of the mtDNA has been dubbed the control region (CR) because it has been shown to be the origin of transcriptional start sites and replication in a number of species (Shadel and Clayton, 1997).

To have high resolution of species differentiation and evolutionary relationships molecular taxonomy is in trend with DNA database. Recently, DNA barcoding has been recommended as an efficient technique to validate species identification using full frame or partial DNA sequences (Hebert et al., 2003) which provide the evolutionary history and relative clades of the species and common ancestor. The word "phylogeny" is derived from the Greek phylon, referring to "clan," or "race," and genesis, which means "origin". Homologous DNA or sequences of proteins are examined in molecular phylogeny to investigate the links between various species or genes. The variation in the obtained sequences points the DNA mutation leading to genetic divergence. These data can be utilized to understand hierarchy in phylogenetic tree. Recent research on the origins and evolution of cone snails used nuclear and mitochondrial markers from Pacific cone snails to rebuild Conidae phylogenies and explore the evolutionary history (Duda and Kohn, 2005; Duda et al., 2008). Ancestral ranges, rates of diversification, dates of divergence, community compositions, and evidence supporting taxonomy hypotheses may all be predicted using a molecular-based phylogenetic tree that has strong support. To clarify the ambiguity and boost the confidence in the outcomes with evidence from many sources is required. In cone snails, species delimitation based only on morphological data is challenging owing to taxonomic obscurity and species complexity brought on by a lack of unique morphological characteristics. The process of identifying the evolutionary connections among species is much improved when morphological approaches and methods based on nuclear and mitochondrial markers are used together.

Venom

Venoms have undergone several evolutionary changes throughout the animal world. These "biochemical weapon systems" is developed either for defence or attacking purpose. Despite the fact that venoms provide model systems for examining predator-prey encounters, molecular evolution, effective conjugation, and new targets for pharmaceutical development, the majority of venomous species are still poorly understood (Casewell et al., 2013).

The *Conus* genus of marine snails is a huge family of carnivorous gastropods that uses a highly sophisticated toxin arsenal for self-defense and prey acquisition. *Conus* venom is studied for over 30 years, the majority of conopeptides range in length from 12 to 35 amino acids. Their exceptional selectivity for macromolecular targets may be partially explained by the vast chemical variety that results from their high frequency of post-translational modifications. They may be roughly classified into two groups: conotoxins, or disulfide-rich conopeptides, which contain two or more disulfides, and conopeptides, or disulfide-poor conopeptides, which have one or no disulfide link (Endean et al., 1974; Gray et al., 1981; Olivera and Cruz, 2001).

In accordance with peptides from known *Conus* spp. few labs, from India, China, Europe, Australia, and Israel, have been deeply involved in drug development projects. An estimated 20% - 30% of all cone species are found in coastal India; nevertheless, few comprehensive research has been published so far. With LC-MS and transcriptome screening the existence of new sulphated peptides has been discovered and identification of peptides has achieved with post-translational modifications.

The venoms of these animals are true pharmacological treasures and with the recent approval of the first cone snail venom-derived drug (Dutertre, 2013). The authorization of the very first marine-derived medicine to treat neuropathic pain has sparked even more interest in marine-derived drug development (Miljanich, 2004). This chemical, a short peptide termed MVIIA or Ziconotide and sold as Prialt, was found as a strong blocker of N-type calcium channels in the venom of a magician cone snail, *Conus magus* (Olivera et al., 1985), approved by the Food and Drug Administration (FDA) for treatment of pain. Several more "conopeptides" that target ion channels, transporters, and receptors are undergoing drug trials for the treatment of chronic pain, as well as myocardial infarction, epilepsy, and neurodegenerative illnesses. As a result, cone snail venoms are prized pharmaceutical commodities (Olivera et al., 2007).

1.3. Objective

Considering the earlier literatures, specifically we observed that Cone snails from the Karnataka coast and the Lakshadweep islands has not been studied with respect to habitat distribution, along with complete morphometrical approach and also there was lack of evidences on phylogenetic relationship. Mainly, conopeptides from *Conus biliosus* venom was not catalogued which is regarded as the research void. To acknowledge this research gap following objectives are proposed:

1. To prepare the checklist and distribution of Cone snail from the Karnataka Coast and the Lakshadweep archipelago.
2. To study the shell morphometric pattern of Cone snails.
3. To establish molecular taxonomic identification of cone snails using mitochondrial genes (COI, 16S rRNA, 12S rRNA) and construction of the phylogenetic tree.
4. Characterization of conopeptides from the *Conus biliosus* venom duct.

CHAPTER 02: Checklist and distribution of cone snails from the Karnataka coast and the Lakshadweep archipelago

2.1. Material and Methods

Sample collection

The Conidae survey and samples were collected during 2021-2023 in the west coast of Karnataka, a few parts of Mangaluru, Udupi, and Uttara Kannada districts of Karnataka, and few islands of Lakshadweep archipelago; Amini, Kadmat, Agathi and Kavaratti islands data are presented. In Karnataka, study area is selected based on the accessible beaches for samplings and the main land water has rough inter-tides and the water turbidity is high, SUCBA dives and snorkelling was difficult. Hence, more of shoreline handpicking method was implemented at different habitats along the sea shores and inter-tides at low tides periods and net trawling method utilized with help of local fishing community. In Lakshadweep archipelago, atoll creates a clear water in island habitat which made sample collection easier in low tides through handpicking and Scuba, snorkelling. During collection both live cone snails, and shells were collected and recorded the observation of habitat.

Distribution analysis

Specimens observed and collected in the survey was identified based on the external morphology of shell, colour pattern and texture; specifications were documented based on Rockel et al., (1995) and recorded in data sheet using M.S. Excel. The α-diversity were calculated using the PAST (version 4) software (Hammer et al., 2001).

2.2. Result

Species account

In Karnataka, 04 species (36 individuals); *C. biliosus*, *C. dictator*, *C. inscriptus* and *C. milneedwardsi* were observed and in Lakshadweep archipelago, 28 species (185 individuals) were recorded. Over all 30 (221 individuals) species were observed from both in Karnataka and Lakshadweep; *C. biliosus* and *C. milneedwardsi* was commonly observed in both the study areas. In this study both live species and live cone snails were observed and recorded.

Diversity indices

The comprehensive checklist showed about 30 species cone snails from the Karnataka and the Lakshadweep archipelago belonging to 02 genus; *Conus* and *Conasprella* belonging to 01 family Conidae, besides only one species i.e., *Conasprella dictator* rest of the records are from a large genus *Conus*. The observation of cone snail is differentiated into α diversity indices and β diversity indices to understand the diversity analysis and species compositions in the habitat. Among these *C. biliosus* is more observed in the Karnataka coast, *C. ebraeus* from Lakshadweep islands were largely observed. *C. betulinus*, *C. eburneus*, *C. flavidus*, *C. frigidus*, *C. lividus*, *C. miles* and *C. arenatus* are also commonly recorded. *C. sanguinolentus* and *C. textile* are least observed individuals.

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Dakshina Kannada (DK) has the highest species richness followed by Uttara Kannada (UKD) region and Udupi (U) being lowest indicating the array of diversity (Table 3) (Fig. 16). But the abundance value is much higher in UKD beaches due to less disturbance and minimal pollution,

this suggests that UKD habitats support larger population of certain species which also reflects on degree of dominance having highest value in the record i.e., 0.649 due to more observation of *C. biliosus*. Whereas, DK region shows balanced species record in terms of abundance.

Species richness estimation through Simson index, Shannon index and species evenness also indicate the measure of DK has higher value followed by U and UDK. A higher Simpson index suggests a more diverse community, so DK shows the most balanced diversity. This result aligns with the Simpson index, reinforcing that DK has the highest diversity in terms of both species richness and evenness. Species in DK and U are more evenly distributed in terms of abundance, while in UKD, certain species are more dominant, leading to less evenness.

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Lakshadweep islands habitats also accommodate larger species of cone snails, Kavaratti (KVT) has the highest species richness, abundance has it hosts wide variety of species this high variation is due to large number of species observed as empty shells from seashore. Agathi (AGT) has highest dominance as occurrence of fewer species are overwhelmingly abundant, while KVT has a more balanced species distribution with lower dominance. Diversity indices such as Simpson index indicates that KVT (0.926), Amini (AMN) (0.886), and Kadmat (KDM) (0.886) have balanced diversity with a variety of species contributing relatively equally to the ecosystem. AGT has a lower Shannon index (1.989), indicating lower diversity. Evenness is highest in KVT, followed by AMN and KDM, with AGT having the lowest evenness. This implies that species are more evenly represented in KVT, whereas AGT shows some species being much more abundant than others.

The distribution of cone snails was recorded with respect to observation in different marine habitats which act as a dynamic ecosystem, also in our study areas species adaptations are observed based on habitats. In Karnataka, *C. milneedwardsi* was observed only in Udupi region. *C. inscriptus* was commonly observed in all three districts. The two species i.e., *C.*

biliosus and *C. dictator* was commonly observed in Dakshina Kannada and Uttara Kannada regions.

Study observed as empty shells in majority of the study area in different habitats such as, rocky shoreline, shallow water sandy bottom, sandy water muddy bottom and algae associated rocky shoreline which are associates with species adaptation. *C. milneedwardsi* has a relatively narrow habitat preference, being found primarily in shallow water muddy bottoms. *C. inscriptus* shows a broader habitat range, associated with both rocky shorelines and shallow water muddy bottoms, suggesting some adaptability to different substrates. The ***C. dictator*** was observed as empty shell from seashore near to rocky shorelines and algae-associated rocks, indicating a preference for rocky or algae-rich environments. ***C. biliosus*** indicates broadest habitat range, associated with all the listed habitats, showing high adaptability across different environments but in Uttara Kannada regions more live specimens were observed live in rocky areas. The width of the connections between species and habitats likely reflects the strength of association or frequency of occurrence in each habitat. Species like *C. biliosus*, which is found in all habitats, may have broader ecological adaptability, whereas *C. milneedwardsi* seems more specialized.

In Lakshadweep islands, majority of species were observed in Kavaratti Island compared to other three island as empty shell collection was observed more in south point shoreline. The species such as *C. miles*, *C. canonicus*, *C. catus*, *C. textile* and *C. violaceus* was observed only in Kavaratti. Whereas, *C. bandanus* was only observed in Amini. *C. betulinus*, *C. chaldaeus*, *C. coffeae*, *C. ebraeus*, *C. frigidus*, *C. lividus* and *C. striatus* were commonly observed in all the islands.

Even in Lakshadweep archipelago the lagoon water hosts variety of habitats such as coral bottoms, rocks associated shoreline, shallow water sandy bottoms, sea grass sandy bottoms and empty shell deposited shorelines were observed. Based on the preferred habitats cone snails species were distributed. The majority of the shells observed as empty shells from seashore also shallow water sandy bottom habitats observations shows equivalent live individuals. Some species are exclusively observed in few particular habitats for foraging and in general they locomote in different habitats. Species such as *C. betulinus* are exclusively observed in sea grass sandy bottom and sandy bottoms. Whereas, *C. lividus*, *C. ebraeus*, and *C. flavidus* observed in rocky intertidal habitat. *C. lividus* is also observed in coral reef platforms including

C. catus, *C. laccadivensis*, *C. zonatus*, *C. mustelinus*, *C. leopardus* and *C. aristophanes*. This indicates that the preference of habitats among live cone snails is dependent on their shell morphometry as associates in selection and locomotion into different habitats.

Rarefied species accumulation curve demonstrates the species richness with sampling efforts. In Karnataka and in Lakshadweep Island sampling areas were selected based on the accessible points; here graphs show number of individuals plotted against sample covered in each area to be saturated, revealing pattern of biodiversity and standardized comparison of species richness. In Karnataka coast the Udupi district and in Lakshadweep Island Amini shows bit of low curve due to numbers of species recorded is less compared to other sampling sites. Apart from that all the other sampling areas describes positive rarefied index in sampling efforts. The plateau (saturation point) suggests the most of the species in the area have been sampled and additional sampling is unlikely to reveal additional species. Further, this can be used for comparing or reassessment of biodiversity of cone snail in the same sampling areas.

Rank abundance curve plots displays species rank (from most to least abundant) on the x-axis and their relative abundance on the y-axis resulting curve provides a visual representation of species abundance and dominance patterns within the group. A steep slope may indicate that species are highly adapted to the environment or have competitive advantages. In Karnataka, Uttara Kannada has more species diversity and in Lakshadweep islands even though Kavaratti has more individuals rare or uncommon species is much recorded in Agathi.

2.3. Discussion and Conclusion

Cone snails as predatory marine molluscs serve key ecological functions, and their venom has great potential for therapeutic applications (Duda, 2008; Dutertre and Lewis, 2013). Thus, there is an interest to explore and understand their distribution, abundance, and ecological adaptations/niches for the purpose of conservation and management. In recent years, researchers have been keenly exploring the distribution and ecological niches of Conidae in the marine habitats around the Indian subcontinent (Ravinesh et al., 2018; Venkitesan et al., 2019; Ravinesh et al., 2022).

As there were no studies on the Conidae from the coastal Karnataka, this study explored the same and recorded four species – *C. biliosus*, *C. dictator*, *C. inscriptus*, and *C. milneedwardsi*.

All four species were previously recorded from the west coast of Indian subcontinent (Puillandre et al., 2014; Ravinesh et al., 2018; Ravinesh et al., 2022). Earlier there were few records of cone snail diversity from Lakshadweep archipelago (Ravinesh et al., 2018) but this study made an attempt to observe the species based on habitat utilization and occurrence by recording 28 species from both live and empty shells of cone snails. All the recorded individuals are from family Conidae (J. Fleming, 1822) and genus *Conus* (Linnaeus, 1758) except *Conasprella dictator* (from Karnataka). Over-all, from both Karnataka coast, and Lakshadweep islands total of 30 species were recorded based on their habitats, it might be unlikely to be their true microhabitats given the fact that majority of them were collected as empty shells which might have simply washed up or settled in the place where they were subsequently observed.

While all listed species are the marked least-concern species in conservation status and *C. aristophanes* and *C. laccadivensis* is listed as data deficient in the IUCN list (Peters et al., 2013); the lack of information on the distribution, ecology, and population diversity are the key obstacles in determining the conservation status of cone snails in general, and the exploration of their venom for therapeutic potential, in particular. The present study has several limitations. It was geographically limited to the accessible marine habitats. The sampling effort was moderate but positive abundance curve, and thus future surveys are likely to reveal additional species.

In conclusion, we have recorded 30 species and no new species were recorded but this study provided the data on the occurrence/distribution of cone snails in the Karnataka coast; Dakshina Kannada shows the highest overall diversity, richness, and evenness, suggesting a more balanced and diverse community structure. Uttara Kannada district, on the other hand, has higher dominance and abundance but lower evenness, indicating that certain species are more prevalent, leading to a less balanced community. Udupi district falls somewhere in between these two sites in terms of diversity and evenness. In Lakshadweep archipelago, Kavaratti stands out as having the highest diversity, species richness, abundance, and evenness, suggesting a well-balanced and diverse ecosystem. In contrast, Agathi has higher dominance and lower evenness, indicating that fewer species dominate, making it a less balanced community. Amini and Kadmat fall in between these extremes in terms of diversity and evenness.

CHAPTER 3: Shell morphometric pattern of cone snails

3.1. Materials and methods

Sample collection and shell processing

From the diversity survey specimens collected from handpicking, net trawling, Scuba dive, and snorkelling; shells were preserved. The intact shells were selected based on the structural integrity; examined for undamaged. Even though the shells are intact but most of the specimens were covered in algae, coral deposits and infected with species of burrowing barnacles. Hence before measurement and analysis, need to clean specimens and make all the sections appear clear. Also, some of the live shells dissected would produce bad odour for several days. So, to clean the shells uniformly standard method of shell cleaning and polishing were followed.

In order to facilitate identification and measurement, specimens were cleaned using a commercial bleaching solutions and water mixture because the collected shells were coated in algae and other organic materials. To eliminate any leftover exterior material, some shells were soaked in 37% hydrochloric acid (HCl) for three to five seconds, and then they were washed with water. This helped to reveal the shell's pattern clearly. Before being cleaned to get rid of the odour, the freshly dissected shells were placed in dirt for two weeks and allowed to dry in the sun. Mineral oil was used to polish the shells' surface for long-term preservation.

Shell Measurements

Based on the literature review of major measurements only the adult shells with taxonomic key features and shell length were consider for measurement. Shells were measured using Vernier Callipers (Kohn and Riggs, 1975). The following variable linear measurements were taken – weight (W in g), shell length (SL), maximum diameter (MD), height of maximum diameter (HMD), aperture height (AH), aperture length (AL), aperture width (AW), height of penultimate whorl (HPW), and spire height (SH). The morphometric parameter was also calculated on the basis of linear measurement and expressed as ratio of linear measures such as relative weight (RW), relative diameter (RD), position of maximum diameter (PMD), relative height of spire (RSH), shape of generating curve (S), rate of whorl expansion (W), relative whorl height (RWH) and convexity (CONV). All linear measurements were in mm.

Based on the measurement minimum – maximum, mean and standard deviation (±SD) was estimated using MS Excel sheet.

Morphometric analysis

The morphometric measures were entered into a Microsoft Excel spreadsheet, the mean (±standard deviation) and minimum-maximum values were calculated. Microsoft Excel was used to create the scatter plots of the morphometric variables (Raup, 1961; Kohn and Riggs, 1975), and the R *corrplot* package (Wei and Simko, 2021) was used to create a Spearman's rank correlation coefficient matrix of the data. Lastly, the R *ggplot2* package was used to do the principal component analysis (PCA) (Wickham, 2016).

3.2. Result

Linear and ratio of linear measurements

Out of overall records only 147 individuals were considered for morphometric analysis i.e., 25 species (n=120) from Lakshadweep archipelago and 04 species (n=27) of Karnataka coast. To ascertain the possible taxonomic use of the proposed morphometric and sculptural traits, the distributions of the shells were also examined.

Unlike other gastropods cone snails are not abundantly scattered in the marine habitat and availability of shell from the shores are also low. The analyzed result of present data compares within the species and differential ratio between species of specimens sampled from same location and also other sampled areas. Ratios are used to represent all continuous variables in order to remove the impact of absolute size discrepancies. Therefore, across the range of sizes taken into consideration, it is anticipated that shell form does not change greatly with size. By statistically examining the dependency relationship between the two parameters in each ratio, the veracity of assumption was examined.

The min-max, mean(±SD) values indicates the overall variables within the species which highlights on the difference of shell growth based on the environmental factors. The linear measurements have relatable variability between species but the ratio of variable is independent. The solid heavy shells *C. betulinus* with 469.4 g and *C. milneedwardsi* 62.5 g are heaviest shells; the small-sized shell *C. coronatus* 2.42 g is the lowest weighing shell. In terms of shell length also *C. betulinus* is of 140.7 mm followed by *C. milneedwardsi* is 136 mm; *C.*

aristophanes 23mm is the shortest solid shell. The widest maximum diameter is 93.9 mm of *C. betulinus*. Height of maximum diameter is dependent on maximum diameter with respect to shell length. Shell aperture is displaying the importance of adaption of cone snail in the habitat for foraging and locomotion. As the narrow and straight aperture cone snails are majorly vermivores habit and less aggressive, whereas piscivores cons snails like *C. geographus* and *C. textile* shells shows wider aperture width comparatively. Number of whorls on the posterior part of the torsion is species specific or varies from species to species. *C. milneedwardsi* has highest with 14 whorl and lowest whorl count is 07 of *C. dictator*, *C. aristophanes*, *C. catus*, *C. chaldaeus*, *C. coffeae*, *C. ebraeus*, *C. frigidus*, *C. geographus*, *C. mustelinus* and *C. zonatus*; including ultimate, penultimate and antepenultimate whorls. Also, number of whorls will not added-up as they grow, it is fixed in number from teleoconch stage.

RW, RD, PMD, RSH, S, W, RWH and CONV are morphometric parameters expressed in the ratios of linear measures, they are interdependent on the direct measurements and independent from species to species. Correlation among these ratios are unpredictable and pace at which the producing curve's size grows with each revolution. Therefore, across the range of sizes taken into consideration, it is anticipated that shell form does not change greatly with size. By statistically examining the dependency structure of the parameters in each ratio, the validity of this presumption was examined.

The scatter plots also provide a good fit for correlation between variable linear measures. MD/SL; y=0.419x, R^2=0.9385 has a positive linear relationship between maximum diameter and shell length, indicating that as the shell length increases, the maximum diameter also increases. HMD/SL; y=0.67x, R^2=0.9935. AH/SL; y=0.7384x, R^2=0.9817. AL/SL; 0.7153x, R^2=0.9869. AW/SL; y=0.0659x, R^2=0.7577. HPW; y=0.0366x, R^2=0.6344. SH; y=0.2435x, R^2=0.8702. All these data represent strong positive correlation. HMD/SL has a strong positive linear relationship with shell length, with a high R^2 value suggesting a near-perfect fit of the regression line to the data.

Scatter plots of Lakshadweep islands, cone snail shells as illustrate the relationships between similar morphological characters; W/SL with positively correlated indicating that larger shells tend to be heavier but R^2=0.3498 value suggests additional factors may influence weight variations, also weight increases as that disproportionately shell length increases. MD, HMD. AH, AL, AW and SH have strong positive correlation but *C. betulinus* and *C. milneedwardsi*

shows little variation has they are large in shell length and maximum diameter compared to other shells which are grouped linearly. HMD/SL indicating a nearly proportional relationship and consistent growth pattern in relation to shell length. Overall, the results highlight that while certain morphological features of cone snails, such as aperture height, aperture length, and maximum diameter, scale linearly with shell length, others like HPW exhibit a more variable relationship, this variability could reflect species-specific or environmentally influenced adaptations, as the height of the penultimate whorl might be less critical for the structural integrity of the shell. Similarly, SH demonstrates a moderate correlation with shell length, suggesting that while spire height generally increases with shell size, it is subject to a degree of variability. Variability in spire height may relate to ecological factors such as habitat type, as different environments might select for different shell forms to enhance camouflage, mobility, or predator deterrence. This differential scaling suggests a combination of allometric and isometric growth patterns among the measured traits.

The estimated Spearman correlation heatmap, based on the overlapping and difference of scatter plots, provides an illustration of the pairwise monotonic relationships between various shell morphometric traits of cone snails. The majority of morphometric traits exhibit strong positive correlations, as indicated by the predominantly darker blue hues in the lower triangle of the heatmap, which reflect Spearman correlation coefficients close to 01. This strong positive correlation suggests that most of these morphological traits increase together, indicating a consistent allometric scaling relationship across the measured dimensions of the shells.

All factors were positively associated, according to the Spearman's rank correlation coefficient matrix of Karnataka coastal cone snail shell morphometric variables, with values ranging from ρ=0.38 to ρ=0.99. the scatter plots of morphometric variables vs shell length. Excellent prediction was demonstrated by factors like HMD (R2=0.994) and AL (R2=0.987) in relation to shell length. Since the samples from C. milneedwardsi deviated from the typical trend, several variables, such AW and HPW, seemed to be more species-specific. Among/within species, the HPW appears to be the least predictable in relation to shell length.

Similarly, in Lakshadweep Island shells display a strong correlation between linear morphometric measurements overall ranged from ρ=-0.004 to ρ=0.99; strong correlation between SL, MD, HMD, AH, Al, AW and SH ranged from ρ=0.53 to ρ=0.99. But the HPW in Lakshadweep shells also shows least predictability in negative range i.e., ρ=-0.007 to ρ=-0.16.

Through this the growth depend characters can be estimated. This correlation matrix indicates that most cone snail shell measurements are positively correlated, with exceptions like HPW, which tends to vary inversely with the overall shell size measurements. This pattern can help in understanding morphometric variations within cone snail species.

In a PCA biplot represents 75.6% of the variation in PC1 and 15.5% of the variance in PC2 were displayed of morphometric measures. All four species' samples had unique clusters, however the C. dictator cluster overlapped or was inside the C. biliosus cluster. C. dictator was less dispersed than other species in the PCA biplot, as can be seen from the reduced variability of morphometric measures. The distribution of long-shelled C. milneedwardsi along the PC1 was one of the variables/arrows projected onto the biplot that showed that SL, MD, and others were the main contributors to PC1. The main contributors to PC2 were the variables HPW, AW, and SH.

Among island shells similar pattern is observed as correlative morphometric measurements showing 73.9% of the variance in PC1 and 13.9% of the variance in PC2. The samples containing n=120 shells clusters 70% at same axis with overlapping ratios, species like *C. betulinus*, *C. leopardus*, *C. milneedwardsi*, *C. laccadivensis*, *C. striatus* and *C. eburneus* has variability and spread-out compared to other species in PCA biplot. Among these most scattered is *C. betulinus* due to its larger shell length and diameter whereas *C. milneedwardsi* all individuals spread-out evenly along PC1. Apart from HPW arrow/variables contributing to PC2 all other characterises contributes to PC1.

Few species show variation in the pattern, and colour of the shell which resembles phenomenon of polymorphisms within a species. In our study *C. biliosus*, *C. eburneus*, and *C. arenatus* shows bit variation in their population but due to lack of molecular evidence currently cannot specify as polymorphisms variation. In *C. biliosus* variation in body colour is common from orange to brown and blackish-brown colour. *C. eburneus* has no change in ground colour but the dot pattern appears in blackish-brown or orange-brown colour. Also, in *C. arenatus* apart from morphometric variation anterior curve had narrow and broad shell width. Some of the damaged shells were not considered in the morphometric study but still evidential inner morphometrical details will help in accessing the over the growth pattern.

3.3. Discussion and Conclusion

Linear measurements were done in an effort to increase the objectivity and quantifiability of using the characteristics of shells as identification traits and to make them repeatable with the development of a mathematical representation. Understanding the morphological variation within cone snail species is essential for exploring their ecological adaptations, evolutionary relationships, and functional morphology. Shell morphology in cone snails is influenced by both genetic factors and environmental conditions, and key measurements such as shell length, aperture dimensions, and spire height can provide insights into species-specific adaptations and phylogenetic relationships and also to identify the juvenile and adult.

Previous records on the morphometric measurements of cone shells of corresponding species from the Indo-Pacific regions (Rockel et al., 1995), Tamil Nadu (Venkitesan et al., 2019), Kerala (Ravinesh et al., 2022), and Lakshadweep (Ravinesh et al., 2018) are comparable to the ranges of values observed in this study. The majority of these characteristics appear to increase proportionately, according to the significant positive correlations across shell morphometric variables; this suggests a consistent allometric scaling relationship across species and individuals (Kohn and Riggs, 1975). Despite the fact that Conidae have similar shell shapes overall (Rockel et al., 1995), morphometric measures are frequently employed to identify species differences.

The present study measured multiple morphological traits of cone snail shells, including weight, maximum diameter, height of maximum diameter, aperture height, aperture width, aperture length, height of the penultimate whorl, and spire height. As an example, *C. betulinus* and *C. milneedwardsi* they show the habitat adaptation as *C. betulinus* has very low spire and adapted more towards sandy bottoms and *C. milneedwardsi* with long spire adapted for various and deeper habitats. All the shells are within the described measurements of earlier studies but in this study overall all the morphometric characterise are addressed and comprehended.

By examining the relationships between these traits, particularly in relation to shell length, we aimed to assess patterns of allometry and isometry within the group. Allometry refers to the differential growth rates of morphological features relative to each other, while isometry suggests proportional scaling. These scaling patterns can reveal whether certain shell features

are under selective pressure to maintain a consistent shape or if they vary adaptively in response to environmental or functional demands.

Overall, the analysis of these morphological relationships adds to our understanding of the growth dynamics in cone snails and provides valuable data for comparing species or populations within the family. Future studies could expand on these findings by exploring how environmental factors, such as substrate type, water depth, or predation pressure, influence these morphological traits. Furthermore, integrating molecular data with morphological observations may reveal whether certain growth patterns are phylogenetically conserved or adaptive responses to specific habitats.

As some of the species were observed and collected in limited numbers; hence, we are uncertain about the generalizability of the comparative analysis findings due to the small number of species and population samples examined. Using a multivariate method and creating an estimation of broad phenetic distance is probably going to be beneficial for a study whose main objective is the taxonomic interpretation of morphometric variations. Only a small portion of the mathematical representations of the numerous secrets of molluscan morphology were to be unrevealed by our investigation.

Chapter 4: Molecular analysis of cone snails using mitochondrial genes (COI, 16S rRNA, 12S rRNA) and construction of the phylogenetic tree

4.1. Materials and methods

Sample collection

Field surveys were conducted (between 2021-23) on the accessible beaches in the three coastal districts: Dakshina Kannada, Udupi, and Uttara Kannada of Karnataka state and Agathi, Amini, Kavaratti, and Kadmat islands of Lakshadweep archipelago. Scuba, snorkelling, Transit and trawling net surveys were performed in various types of coastal marine habitats from intertidal to subtidal bottoms. Identifications of the collected specimens were based on the description of the shell morphology (Rocket et al., 1995). The foot tissue of the live specimens was preserved in 90% ethanol for molecular analysis.

DNA extraction and PCR amplification

The genetic material was obtained from C. biliosus foot tissue. Using the CTAB procedure (Doyle and Doyle, 1987), DNA was extracted from 40 mg of tissue. For a few species, Origin's customized marine premium kit was utilized, yielding around 500 ng/µl of DNA. The universal primers were used to amplify the genes for mitochondrial cytochrome oxidase subunit I (COI), 16S rRNA, and 12S rRNA. 3µl of DNA, 1.5µl of each primer, 13.5µl of Takara master mix, 1.5µl MgCl2, and 9µl of PCR gradient water were used in the 30µl PCR reactions. Amplification reactions were first denatured for 4 minutes at 94°C, and then they were denaturated for 40 seconds using a 35-cycle process. In the mid-stage, COI, 16S, and 12S were annealed for 40 seconds at 51°C, 52°C, and 54°C; in the final stage, they were extended for 1 minute at 72°C, and in the final round, they were extended for 5 minutes at 72°C (Laxmilatha et al., 2021). A NanoDrop spectrophotometer (ThermoFisher Scientific Pvt. Ltd.) was used to measure the genomic DNA concentration and yield of the PCR products quantitatively. DNA and PCR products were assessed qualitatively using 0.8% agarose gel electrophoresis and 2% agarose gel electrophoresis, respectively.

mtDNA gene Sanger sequencing:

Utilizing thermal cycler, PCR products are sequenced with labeled ddNTPs and dNTPs with single primer as cycle sequencing involving initial denaturation at 95°C for 1:15 sec, denaturation at 95°C for 45 sec, annealing at 50°C for 5 sec, extension at 58°C for 2:50 sec and final hold at 4°C. The reaction is prepared with Big Dye V3.1, 5X sequencing buffer, and 5M betaine and purifying PCR products using EXO SAP enzyme.

A clean-up step is implemented before capillary electrophoresis to filter fluorescent ddNTPs, salts and other impurities. And ethanol/ETDA precipitation technique is employed to combined with 3M sodium acetate and 125mM EDTA along with alcohol for about 10-20 min incubation followed by centrifugation at 6580 rpm for 30 min. Supernatant is discarded and remaining pellet is washed with 70% alcohol; later Hi-Di formamide is added to pellet and introduced into instrument.

To conduct sequencing reactions, capillary electrophoresis is used as arrays into genetic analyzer with 96-well plate. In this process to attain single-nucleotide resolution, tagged chain-terminated fragments are segregated according to their length. Sequencing software is used to translate fluorescent peaks into each nucleotide sequence and produce an ab1. file (Hebert et al., 2003; Simon et al., 1994 and Kocher et al., 1989).

Phylogenetic analysis

The top-hit sequences were utilized as references after the acquired sequences were BLASTed against the NCBI database. MUSCLE in UGENE Unipro software was used to align multiple sequences, and MEGA7 software's Kimura-2-parameters (K2P) model and maximum likelihood (ML) approach were used to build the phylogenetic tree (Kumar et al., 2016). There was a thousand bootstrap sampling.

4.2. Results

Only in Karwar, Uttara Kannada district live *Conus biliosus* (n=2) were observed and collected from rocky shorelines and algae-covered rocks and remaining species were collected live from Lakshadweep archipelago.

For the successful quality sequence obtained maximum likelihood phylogeny tree was reconstructed; for *C. biliosus* separate COI partial gene-based phylogeny tree was made to see the variation as it was the only species observed live in the Karnataka coast in this study and for the Lakshadweep Island species coding gene COI and non-coding gene 16S rRNA and 12S rRNA based phylogeny tree was reconstructed

Mitochondrial cytochrome oxidase subunit 1 (COI) gene-based ML phylogenetic tree of *C. biliosus* from Karnataka

Two sequences of *C. biliosus* were acquire and submit to NCBI, the partial sequences of 12S rRNA, 16S rRNA, and mitochondrial COI. According to the phylogenetic tree based on mitochondrial COI sequences, the Karnataka C. biliosus samples PQ390234 and PQ392002 were different low variation and were distinct, but they belonged to the same clade as other *C. biliosus* samples from the Indo-West-Pacific area (KJ549870.1 and KJ550138.1) (Puillandre et al., 2014). A distinct outgroup was generated by C. shikamai and other species. The NCBI database does not have any appropriate quality sequences, hence the 12S and 16S rRNA sequences were not used to build the phylogenetic tree.

Mitochondrial cytochrome oxidase subunit 1 (COI) gene-based ML phylogenetic tree

The partial sequences mitochondrial COI gene was trimmed and aligned threshold resulting in phylogenic tree reconstruction of monophyletic model. With variation of 0.0067 to 0.0259 nodal values of two major branch length is obtained with frequency around 96-100 boot strap values which is useful to identify and evaluate species-level using single gene tree. Sequence from 15 species shows very low variation of about >5% between them and cladding of species is similar to their foraging and environmental adaptive behaviour. This supports a monophyletic grouping and a common ancestor in the evolutionary lineage, species might have diverged recently and thus haven't accumulated significant genetic differences. Environmental adaptations and foraging strategies could drive parallel evolutionary trajectories, resulting in clades with low interspecies variation.

Mitochondrial 16S rRNA and 12S rRNA genes-based ML phylogenetic tree

These clades were likewise built using the 16S rRNA and 12S rRNA gene concatenation of 15 species, resulting in greater support cluster and less variation 0.00166-0.0028 but overall >1.5%. Boot strap values are near to 100 but for some species there are bit of variation on an average population.

The pairwise distance matrix analysis shows the similarity of divergence between species. The intraspecific distance between different individual of same species values is low which represent that the it belongs to the same population. The phenotypic difference is between overall species is 0.0 to 0.2 intra and inter-specifically for coding gene COI. The concatenated non-coding genes 16S rRNA and 12S rRNA shows very low variation i.e., 0.0 to 0.1. This study observed that the various gene segments differ in their capacity to elucidate the links between members. We predicted that noncoding mitochondrial 16S and 12S mitochondrial markers would be better at differentiating genetic variants among evolutionarily distinct units than mitochondrial protein-coding COI markers because of the fast pace of gene change in *Conus*. Following all the sequences were examined for quality and verified sequences were deposited in NCBI database.

4.3. Discussion and Conclusion

Cone snails are a large varied group, as demonstrated by molecular phylogenetic analysis, despite their general phenotypic similarity, which was used to argue their inclusion in a single species until recently. Cone snail speciation arises from several evolutionary processes since the group may fit into a number of theories of speciation that are related to geography or ecology. Cone snails are among the most varied families of marine invertebrates, and this tendency to speciate after numerous evolutionary processes is probably one of the main reasons for this.

Marine molluscs have a high degree of species variety and morphological plasticity (Doebeli & Dieckmann, 2003). Both a response to shifting environmental factors and cumulative genetic diversity caused by the discontinuity of gene flow among populations can lead to phenotypic alterations (Palumbi 1994; Doebeli & Dieckmann, 2003; Duda and Kohn 2005).

Additionally, it is argued that because the majority of research conducted over the past three decades have concentrated on species that are part of a small number of lineages, the pharmacological variety of the peptides detected in the cone snails' venom gland has been overestimated (Puillandre et al., 2012a, 2012b).

The phylogenetic analysis based on mitochondrial COI of live *C. biliosus* specimens collected in this study showed them in a *monophyletic* group with *C. bilious meyeri,* a southern most subspecies found in Indian marine habitats (Puillandre et al., 2014). Given large number of samples, genetic markers are a preferred tool to explore the evolutionary process of speciation and divergence of venoms in cone snails according to ecological adaptive radiation (Duda and Palumbi, 1999; Duda, 2008).

Among the collected samples of each *Conus* species, the average sequence lengths of COI (650 bp), 16S (450 bp), and 12S (380 bp) were determined. 15 species of cone snails were divided into distinct subclades using both protein-coding COI and non-coding markers, and their maximum likelihood phylogeny was reconstructed using a variety of mitochondrial markers. 30 individuals of 15 *Conus* species had molecular signatures created for them in the current study. When creating the maximum likelihood phylogenetic tree, species with comparable shell

shape and feeding habits were found to cluster together as specimens collected were majorly piscivorous.

Genera delimitation through morphological data is highly challenging because of the presence of species complex due to the absence of obvious morphological uniqueness and the being of morphological intergradation among recognized species. Based on this *C. laccadivensis* was described by segregating from *C. distans* population in Lakshadweep (Laxmilatha et al., 2021), even our sample of *C. laccadivensis* shows 99% similarity with the recently described population from the same geographical area. Other species also identified similarly and resembles as per earlier literature subgenus groups such as *C. frigidus* (*Virgiconus*); *C. laccadivensis* (*Fraterconus*); *C. catus*, and *C. striatus* (*Pionoconus*); *C. eburneus*, and *C. tessulatus*, (*Teselliconus*); *C. biliosus* (*Splinoconus*); *C. arenatus* (*Puncticulis*); *C. miles* (*Rhizoconus*); *C. leopardus* (*Lithoconus*); *C. lividus* (*Lividoconus*); *C. betulinus* (*Dendroconus*); *C. ebraeus* (*Virroconus*); and *C. zonatus* (*Stephanoconus*) (Puillandre et al., 2014).

However, the manner of speciation has been impacted by genetic and geographic variables (Palumbi, 1994). Genetic study of quickly developing mitochondrial codes (COI) genetic markers, gradually progressing non-coding (12S rRNA, 16S rRNA) hereditary markers coupled with nuclear markers are thus appropriate techniques for determining the genetic mechanism of speciation.

In Conclusion, this study did not observe any large variation as novel species. Molecular confirmation of species in this study shown has very less dissimilarity in intraspecific and interspecific population and all the species are similar to earlier identified species.

CHAPTER 5: Characterization of conopeptides from the *Conus biliosus* venom duct

5.1. Material and methods

Sample collection and venom extraction

or this investigation both dead shells and live *Conus biliosus* were sampled from the intertidal rocky shoreline and algae associated rocky habitats from Karwar, coastal line of Karnataka through handpicking method in shoreline transit survey during low tide. The collected specimen was comprehensively compared for taxonomic confirmation and identified through external morphology using a field guide (Rockel et al. 1995). Further live specimen was dissected and venom apparatus i.e., venom gland, duct was stored in 70% ethanol for mass spectrometry.

For intact and reduction mass spectra analysis, samples were prepared with venom duct, which was grinded with a micro pestle and made into a fine paste with an acetonitrile and methanol mixture (50:50) as organic extraction. centrifuged the mixture, and supernatant was collected from the dissolved crude venom for LC-MS injection.

Transcriptome assembly

Performed a literature search and found a *Conus* biliosus transcriptome in the NCBI database (Accession No: PRJNA280558 ID: 280558). This venom duct transcriptome was generated using the Ion Torrent PGM system. The metadata shows that the sample was collected from Porbandar coastal region, Gujarat, India. We used this publicly available RNA-Seq data to identify the prospective conopressin peptide sequence and compare it with the results of mass spectrometry. Downloaded FASTQ reads were subjected to quality check using autoadapt package (a combination of FastQC and cutadapt) (Martin, 2011). This tool automatically detects the adaptors and primers present in a FASTQ file and trims those regions. The pruned reads were then assembled using rnaSPAdes using the iontorrent flag. We used rnaSPAdes due to its ability to assemble reads generated on the Ion Torrent platform (Bushmanova et al., 2019). The resulting contigs were input into the Conodictor 2.0 software to identify and classify prospective conopeptide sequences (Koua et al., 2021). A manual search was performed to identify the peptide sequences discerned using mass spectrometric analysis of our sample.

The search was unsuccessful due to the over trimmed ends. Hence, we performed transcriptome assembly using Trinity v2.15.1. (Grabherr et al., 2011; Haas et al., 2013) Conodictor 2.0 software was used to identify and classify prospective conopeptide sequences. The resulting contigs were manually searched for the peptide sequences discerned using mass spectrometric analysis of our sample. For comparison, a full length conopression-conophysin sequence was used to extract homologues from the Uniprot database. A total of 40 sequences from different cone snail species were obtained and aligned using UGENE Unipro software (Okonechnikov et al., 2012). A Neighbour –Joining tree was constructed to observe clustering pattern.

Mass spectrometry analysis

Isolation of venom toxins

Crude venom peptides from were extracted using 50% acetonitrile-water solvent from venom ducts of *Conus biliosous*.

Reduction and alkylation process

2µg of crude extracted venom peptides from *Conus biliosous* was reduced with 10 mM of dithiothreitol (DTT) and incubate at 60°C for 30 min and further the cysteine residues were alkylated with 20 mM of iodoacetamide (IAM) at room temperature in dark condition for 30 min in 50 mM of ammonium bicarbonate, pH 7.8.

LC/MS analysis

The resultant reduced and alkylated peptides and crude intact venom peptides were injected into EASY nLC 1200 UHPLC system (Thermo Scientific, Germany) equipped with Thermo Easy Spray Column; Acclaim™ PepMap™ RSLC C18 (3 µm, 100 Å x 75 µm x 50 cm) coupled to orbitrap mass spectrometer (Orbitrap Fusion™ mass spectrometer, Thermo Scientific, San Jose, California, USA). Peptides were eluted with a gradient of 10-50% of solvent B over 60 min using a flow rate of 300 nl/min, where solvents A and B consisted of water and acetonitrile with 0.1% FA, respectively.

Mass spectrometric data analysis

The mass spectrometric data were visualised by Thermo Xcalibur qual browser and the peptides were analysed by *de novo* peptide sequencing method. Additionally, the precursor peptide identification was carried out by BLAST analysis.

5.2. Results

One mature conopressin/conophyssin and two linear conopeptide was identified using de novo sequencing method.

Transcriptome analysis

The mRNA data of *C. biliosus* found along the coast of India were examined to identify the linear short conopeptide and conopressin precursor sequences. This data represent variation of conopeptide by performing proteome analysis on the aqueous extracts of *C. biliosus* venom duct in order to investigate potential active conopeptides. Annotated conopressin precursor sequence was used for the BLAST analysis through Uniport database. Multiple alignment of linear conopeptide and conopressin of *C. biliosus* i.e., CYIRNCP. As per Kumar et al., 2020 the residue number followed by the single letter code in parenthesis indicating the position(s) of mutation(s) may be used to define further differences. Cys-Tyr-Ile-Arg-Asn-Cys-Pro with respect to references sequences position 2 (Tyr and Phe), position 4 (Arg, Thr, Gln and Gly) and position 7 (Pro and Leu) has few variations. The last two restudies will vary based on species specific.

The Clustal-omega alignment also shows linear conopeptide of 17 residues with long open chain i.e., Phe-Gly-Pro-Asp-Met-Gln-Gly-Met- Gln-Ala-Met-Pro-Gly-Ala-Met-Pro-Gly represented major variability of amino acids in all the positions in the sequences except position 1 (Phe) remains common in the MLRLI genes sequences being the mature linear conopeptide. Position 2 also shows less dissimilarity (Asp and Gly), 3 (Pro, Gln and Gly), 4 (Asp, Ala and Gly), 5 (Met, Leu and Ala), 6 (Gln, Met and Leu), 7 (Gly, Pro, Ala and Thr), 8 (Met and Ile), 9 (Gln and Pro), 10 (Ala, Ser, Asn, Ile and Ala), 11 (Met and Leu), 12 (Pro, Gln, Val and Ala), 13 (Gly, Pro and Asp), 14 (Ala, Met, Leu and Val), 15 (Met, Gln and Pro), 16 (Pro, Gly and Lys) and 17 (Gly, Met and Arg). Among this position 7, 12 and 14 has four amino acid variations and only position 10 shows highest dissimilarity with five variable amino acids. This long chain linear conopeptide is not a standard peptide chain and shows most viability from species to species and this may be due to environment specific.

The other short linear conopeptide in i.e., Phe-Leu-Pro-Phe-Asn-Pro, has similar amino acids arrangement with all the references species compared with *C. biliosus* amino acids positions and as per BLAST results received from the database.

Conopeptide identified through LC-MS

The LC-ESI-MS analysis of the *C. biliosus* crude venom extract performed on a Orbitrap Fusion mass spectrometer. *De novo* sequencing of toxin polypeptide from *C. biliosus* provides evidence of sequence variation and post-translational modification. The mass spectrometric analysis of from reduced and alkylated identified CYIRNCP with a mass of 865.38 Da [m/z of 491.72 (+2)] and its intact disulfide bonded peptide in crude sample showed the evidence of mature truncated conopressin peptide characteristics. The conopressin class of peptides are synthesized from the proneurophysin precursor by selective proteolysis. Cone snail toxins consist with highly rich cysteine disulfides connectivity.

The long linear peptide with 17 residues with 1721.72 Da [m/z of 861.86 (+2)] FGPDMQGMQAMPGAMPG, this linear conopeptide has a mixture of polar (Gln, Met) and minuscule nonpolar (Ala, Gly, Pro) residues. Given their large concentrations, proline and glycine are probably responsible for the peptide's structural flexibility. A methionine residue (Met) may increase the peptide's oxidation susceptibility and cause a mass shift when exposed to oxidizing environments. The absence of cysteine residues in the sequence suggests that it is a non-cystine framework peptide. The peptide's anticipated molecular weight is in line with the doubly charged ion detected at 861.86. For peptides of this size, the emergence of a +2 charge is usual; efficient ionization is achieved by protonating both the N-terminus and one extra site, which is most likely a basic or amide-containing residue.

Short linear conopeptide FLPFNP of 733.37 Da [m/z 734.39 (+1)]. One positively charged conopeptide sequence with polar and hydrophobic residues alternating in it. The hydrophobicity and aromaticity of the peptide are greatly enhanced by repeated appearances of phenylalanine (F) and proline (P) at positions 1 and 3, respectively. A further potential function of the aromatic ring in conopeptides related to receptor binding or channel regulation is to maintain binding contacts in receptor pockets. Our finding might provide the insight of molecular analysis of venom composition in *C. biliosus* and understand the diversity in the toxins.

Structural analysis of identified conopeptides

The ball-stick predicted model of conopeptide (amino acid residues) are cyclic structures, these peptide forms a cyclic loop, likely due to a disulfide bond between Cys-1 and Cys-6. The yellow connection between these residues is a disulfide bridge, common in stabilizing the 3D structure of small peptides. The amino acid residues Cys (Cysteine)**:** Residue 1 and 6,

connected via a disulfide bond; Tyr (Tyrosine)**:** Residue 2, an aromatic amino acid; Ile (Isoleucine)**:** Residue 3, a hydrophobic amino acid; Arg (Arginine)**:** Residue 4, a positively charged, basic amino acid; Asn (Asparagine)**:** Residue 5, a polar amino acid with an amide side chain; Pro (Proline)**:** Residue 7, a cyclic amino acid that introduces bends in structures. The side chains of each amino acid are extended from the backbone and are shown in distinct orientations. Functional groups like hydroxyl (-OH) in tyrosine, amide in asparagine, and guanidinium in arginine are visible. The presence of a cyclic disulfide bond suggests that this peptide has a constrained and stable structure, often seen in bioactive peptides like toxins, hormones, or antimicrobial peptides.

A long chain linear conopeptide with multiple amino acids labelled and a potential secondary structure indicated by the spatial arrangement, forming a polypeptide backbone. The amino acid residues PHE-1 (Phenylalanine): An aromatic amino acid at the N-terminal end; GLY (Glycine)**:** A small, flexible amino acid that appears at positions 2, 7, 13, and 17; PRO (Proline)**:** Found at positions 3, 12, and 16, it introduces kinks or bends in the peptide chain; (Aspartic ASP acid)**:** A negatively charged amino acid at position 4; GLN (Glutamine)**:** A polar amino acid at position 6**;** MET (Methionine)**:** A sulfur-containing amino acid at positions 8 and 11; ALA (Alanine)**:** A small, hydrophobic amino acid at positions 10 and 14. The red atoms represent oxygen (e.g., in carbonyl and carboxyl groups), while the blue atoms represent nitrogen (e.g., in amides). The three-dimensional folding of this peptide shows specific bends and folding due to interactions like disulfide bonds between cysteines and possible hydrogen bonding or van der Waals interactions between residues.

The short linear conopeptide illustrates six amino acid residues arranged sequentially and connected via peptide bonds (gray backbone). Amino acid residues Phe-1 (Phenylalanine)**:** An aromatic amino acid at the N-terminal end; Leu-2 (Leucine)**:** A hydrophobic amino acid with a branched aliphatic side chain**;** Pro-3 (Proline)**:** A cyclic amino acid that introduces rigidity or bends in the peptide chain; Phe-4 (Phenylalanine): Another aromatic amino acid, contributing hydrophobicity and potential π-π interactions; Asn-5 (Asparagine): A polar amino acid with an amide side chain, capable of forming hydrogen bonds; Pro-6 (Proline): The C-terminal residue, introducing another bend or kink in the structure. The backbone is shown in grey, with alternating nitrogen (blue) and oxygen (red) atoms. The side chains extend outward, showing distinct characteristics Aromatic groups from Phe**-**1 and Phe-4; Polar side chain in Asn-5 (amide group); Hydrophobic aliphatic chains in Leu-2**;** Cyclic rings in Pro-3 and Pro-6. The

structure displays a bent configuration due to the presence of proline residues, which are known for restricting flexibility and introducing structural constraints. The orientation of the side chains and backbone suggests potential interactions, such as hydrogen bonding or hydrophobic packing.

5.3. Discussion and Conclusion

A great variety of short peptides with pharmacological activity may be found in *Conus* spp. venom. Disulfide-poor conotoxins make up a small portion of this venom mixture. Cysteine-poor conopeptides are less studied than their cysteine-rich counterparts because an even smaller fraction only contains one disulfide link. There is a concentrated effort to find and characterize new conotoxins as a result of the conotoxins unique potency and selectivity profiles for a variety of neuronal targets, which have made several of them useful as research tools, drug leads, and even therapies. In this article based on the mass spectrometry de novo analysis we identified the linear peptides and conopressin superfamily from *C. biliosus* which illustrate the bio-active conopeptide of therapeutically significant conopeptide.

Conopressin was initially separated from the venom of *C. geographus* and *C. striatus*. Conopressin-S was the name given to the *C. striatus* peptide, and conopressin-G to the *C. geographus* peptide CFIRNCPKG*. These peptides were shown to exhibit grooming and scratching behaviour in mice, following intracerebral injection (Cruz et al., 1987). In our study similar conopressin was identified as CYIRNCPKG* from *C. biliosus* observed in Karnataka. In most of the other species *C. monile*, *C. lividus*, *C. loroisii*, *C. frigidus*, *C. linavati*, and *C. bayani* (Kumar et al., 2020 and Dutertre et al., 2008) majority of the conopressin sequence identified as the basic Arg or commonly observed in the position 4. The only exception is the venom of *C. tulipa*, which contains conopressin-T (Con-T), which bears the sequence of CYIQNCLRV, peptide which represent Vasopressin family, as there are similar to conopressin.

The additional restudies such as C-C from position 1 and 5 and the signature placing of conopressin in single disulphide bond (Nielsen et al., 1994), the last residual bond position 8 and 9 are not strong enough majority of the sequences in post translation studies they are loosen which are one of the most dissimilarity amino group among other species. The residue number and the one-letter code in conjunction that indicates the positions of the mutations may be used

to characterize further variants. In general, the conopressin full gene mRNA sequence is from MTRS group.

In this study two linear peptides from *C. biliosus* were identified FGPDMQGMQAMPGAMPG and FLPFNP, these linear peptides are group from MLRLI (B2 superfamily) of full-length gene identified by mass spectrometry is large cysteine-free class of about 140-160 residues in length. The cysteine-free acyclic conopeptides are least studied compared with conopressin or complex conotoxins. A distinctive feature of neuropeptides is the C-terminus amidation, which is frequently present in mature acyclic peptides that have undergone post-translational modification. In recent studies on linear conopeptide has also shown C-terminus amidation and N-terminal conversion from *C. monile* and *C. betulinus* (Vijayasarathy et al., 2023). But the MLRLI superfamily also represent Cysteine free linear conopeptides. The 17 residues amino group from *C. lenavati* is similar to *C. biliosus* with very few positions dissimilarity on the last positions. Apart from that the other species from the multiple sequence alignment shows variation in all the position of amino acid apart from Phe in position 1 as common amino acid. The other short precursor conopeptide sequence FLPFNP observed has 100% identification similarity between other species from multiple sequence alignment which can be considered as common acyclic linear conopeptide.

Cysteine-free peptides are found in the venom of several cone snail species. Different proteolytic breakdowns of precursor proteins and post-translational modifications enhance the size and variability of acyclic peptide catalogues. Several vertebrates and invertebrates have been found to include neuropeptide equivalents in a variety of cone snail venoms. To identify the biologically relevant targets of acyclic conopeptides along with investigate their biological effects, more research is required. Research should be encouraged since acyclic sequences are much easier to synthesize than many disulfide-bonded conotoxins (Dutertre et al., 2008; and Van Kesteren et al., 1995)

We are uncertain of this conopeptide activity in defensive or attacking injection of venom. This is also specific to evolutionary questions such as measuring divergent in venom and genetic distance.

Summary

This study is focused on the overall understanding the habitat vise distribution, morphonology, molecular identification of cone snail species along with active pharmacologically important conopeptide cataloging from lesser studied species i.e., *C. biliosus*. We were able to collect the samples from accessible beaches from selected study area across the Karnataka coast from districts of Dakshina Kannada, Udupi, Uttara Kannada and Agathi, Kavaratti, Amini and Kadmat islands of Lakshadweep archipelago.

1. The distribution and checklist of the cone snails represent the *Conus* spp. which are commonly observed in both study area. Overall, 30 species were recorded four from the Karnataka coast and 28 species from Lakshadweep archipelago in which two species are repetitive i.e., *C. biliosus* and *C. milneedwardsi.* Majority are from genus *Conus* and only one species *Conasprella dictator* belongs to genus *Conasprella*. In Karnataka, Uttara Kannada district represents high species richness and in Lakshadweep islands Kavaratti show more species richness. Cone snails have utilized multiple types of habitats in the marine environment generally seen in sandy bottom shallow water and few were observed in deep waters. Some of the species such as *C. biliosus, C. zonatus*, *C. laccadivensis*, *C. ebraeus*, *C. tessulatus* and *C. mustelinus* are adapted to specific habitats like coral reef platform and rocky shorelines.

2. Shell morphometry mathematically represents the external shell morphology and pattern variation. Most of the adult shell growth are known from earlier studies and records from this study is within the estimated range by which adult and juveniles can be observed. *C. betulinus* being the heaviest shell, *C. coronatus* the low weighing, *C. milneedwardsi* is the longest shell and C. aristophanes is the smallest shell recorded in this study. Relatively all linear measurements are dependent on shell length (SL) and maximum diameter (MD) but je ratios of linear measurements are independent even though derived from linear values. *C. milneedwardsi* forms outliner is most of the correlation as it is the longest among the records. Height of penultimate whorl (HPW) and spire height (SH) are the most independent of all values in the linear analysis and shows negative correlation as other linear exhibits positive correlation.

3. In molecular analysis, only *C. biliosus* was analyzed from Karnataka as no other live individuals were observed and also very low data available in database for construction of phylogeny tree. From COI gene analysis *C. biliosus* shows close relationship with *C. biliosus meyeri* from Indo-Pacific waters and this *C. biliosus* molecular data from this study is the third sequences deposited in the NCBI database. Cone snails of Lakshadweep islands also shows less variation in the mtDNA sequences are with high bootstrap values. Distance matrix also shows that 0.0 to 0.2 COI and 0.0 to 0.1 16S rRNA and 12S rRNA variation was observed. All the species are very close the earlier describe species and this method helped in identification and conformation of species.

4. Transcriptome assembly and de novo LC-ESI-MS analysis reveals major three matured conopeptides i.e., Conopressin from MTRS super family and one long chain acyclic linear conopeptide and short chain acyclic linear peptide from MLRLI super family which are active conopeptide an can be analyzed for threptic approaches.

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Publications

1. B S Chandan and Mohammed S Mustak. Report on Conidae (Mollusca, Gastropoda) from Karnataka Coast of India (2024). (*In communication*).
2. B S Chandan, Rajdeep Das, Jaison J S and Mohammed S Mustak. Identification of mature conopressin and linear peptide from *Conus biliosus* venom. (*In submission stage*).
3. B S Chandan and Mohammed S Mustak. Distribution, Morphometry and Molecular phylogeny of Conidae from Lakshadweep archipelago. (*Under preparation*).

Conferences (Oral/poster presentation)

1. Participated and Paper presented in National Conference on "Molecular phylogenetics of the genus *Conus* (Family-Conidae) inferred using mitochondrial DNA markers". Organized by St. Aloysius College (Autonomous), Mangaluru. 10th May 2022.
   Note: Won First place in paper presentation category.
2. Participated and poster presented in the 09th Indian Peptide Symposium & INDIA PEPTIDE SHOW on "Characterization of *Conus biliosus* Venom (conopeptide) using Mass Spectrometry-based Proteomics". Organized by INDIAN PEPTIDE SOCIETY at BITS, Pilani - K K Birla Goa Campus. 23rd to 25th February, 2023.
3. Participated and paper/oral presented in the 04th DST-SERB School on Chemical Ecology on "Identification of Conopressin from *Conus biliosus* venom-Conopeptide". Organized by CES, IISc, Bangalore. 08th to 22nd of July 2023.
   Note: Won second place in paper presentation category.
4. Participated and paper/oral presented in the BIOTRENDCON on "Molecular phylogeny of Conus biliosus and their Venom (Conopeptide) Bioprospecting". Organized by Department of Biotechnology and International Conclave on Engineering Sciences & Technology, P. A. College of Engineering, Mangaluru. 23th to 24th of April 2024.

Workshops

1. Participated in webinar on "How to read scientific literature?" and completed online evaluation. Organized by National Centre for Cell Science and MANAV-Human Atlas Initiative, I1SER, Pune. 19th November 2021. Certificate ID: ISYYCS-CE000019.

2. Participated in workshop on “Research Methodology”. Organized by Department of Bioscience, Mangalore University. 18th December 2021.
3. Participated in Six days workshop on “Research and Publication Ethics”. Organized by Department of Education, Mangalore University. 18th February 2022 to 23rd February 2022.
4. Participated in “High end workshop on Chromatography Techniques: HPTLC and HPLC”. Organized by SERB, India and Manipal School of Life Science, Manipal Academy of Higher Education, Manipal. 04th to 10th July 2022.
5. Participated in “Seven Day National Level Training Programme” of DST STUTI 2022. Organized by DST-PURSE Instrumentation Centre, CART and MHRD-RUSA NMR Instrument Centre, Mangalore University in collaboration with Shivaji University, Kolhapur, Maharashtra. 14th to 20th November 2022.
6. Participated in training program on National Intellectual property awareness mission organized by Dept. of Biosciences, Mangalore in February 04, 2023.
7. Participated in 4th SERB School of Chemical Ecology 2023, Organized by DST at CES, IISc, Bengaluru from 8th to 22nd July 2023.
8. Participated in “Webinar Training on QGIS Fundamentals”. Organized by TGIS Online Platform, Ahmedabad (Gujarat). 23rd to 24th September 2023.
9. Participated in “QGIS Advance Applications”. Organized by TGIS Online Platform, Ahmedabad (Gujarat). 28th to 29th October 2023.
10. Participated and attended the digital modules of One-month international workshop on “Genome Informatics (6th Edition)” organized by Decode Life. Certificate no.: DECODE-G6/81. 06th January to 04th February 2024.

(Dr. Mohammed Shafiul Mustak B.)
Signature of the Research Supervisor

(Mr. Chandan B S)
Signature of the Research Scholar